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(71) Applicant *(for all designated States except US)*: AGRICULTURAL RESEARCH COUNCIL [ZA/ZA]; ARC House, Park Street, Hatfield, 0083 Pretoria (ZA).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: DE BRUYN, Engela, Elizabeth [ZA/ZA]; 761 Nebo Crescent, Faerie Glen, 0043 Pretoria (ZA). BOTHA, Adriaan, David [ZA/ZA]; Plot 107, Leeufontein, 0486 Pretoria (ZA).

(74) Agent: KAHN, Craig, Glen; Adams & Adams Pretoria Office, Adams & Adams Place, 1140 Prospect Street, Hatfield, 0001 Pretoria (ZA).

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(54) Title: VACCINE COMPRISING A NON–TOXIC IMMUNOGENIC DERIVATIVE OF *CLOSTRIDIUM BOTULINUM* TYPE D NEUROTOXIN

(57) **Abstract**

The invention provides a non–toxic immunogenic derivative of *C. botulinum* type D neurotoxin or an immunogenic fragment thereof, said derivative or fragment carrying at least one mutation in its amino acid sequence, not found in wild–type D neurotoxins. The invention also extends to a nucleotide sequence comprising a recombinant DNA fragment characterised in that said DNA fragment encodes a non–toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof.

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VACCINE COMPRISING A NON-TOXIC IMMUNOGENIC DERIVATIVE OF *CLOSTRIDIUM BOTULINUM* TYPE D NEUROTOXIN

The present invention relates to immunogenic derivatives of *Clostridium botulinum* neurotoxin, DNA encoding such derivatives, bacterial expression systems comprising DNA encoding such derivatives, non-*Clostridium botulinum*-based bacterial expression systems expressing immunogenic botulinum neurotoxin derivatives, vaccines for combating *C. botulinum* neurotoxins, methods for the preparation of immunogenic derivatives of *C. botulinum* neurotoxin and to methods for the preparation of vaccines for combating *C. botulinum* neurotoxin.

Clostridium botulinum is a species of the large bacterial genus *Clostridium*. Bacteria belonging to this genus are spore-forming anaerobic Gram positive bacilli. The species *C. botulinum* can be sub-divided into types and the different types produce several toxins, e.g. such as types A, C, D and E. Types C and D are generally pathogenic to cattle, sheep, pigs, man, goats, donkeys, fish, horses, mules, dogs and birds. Botulinum neurotoxin (BoNT) causes botulism poisoning which commonly occurs in cattle and sheep in South Africa, Australia, South America and various other countries. Pathogenesis or poisoning usually results after the ingestion of carrion or decomposed material contaminated with BoNT produced by *C. botulinum* bacteria. Clostridial neurotoxins are one of the most toxic substances known and usually result in the death of the affected or poisoned animal. The target sites of the BoNT are the

cholinergic nerve endings of neurons in the animal. The BoNT affects these nerve endings by acting as a zinc-dependent endoprotease to cleave polypeptides that are necessary for exocytosis of neurotransmitter containing vesicles. Cleavage of these polypeptides leads to blockage of transmitter release which results in paralysis and thereafter usually death of the affected animal. BoNT is a protein with an approximate molecular mass of 150 kDa. BoNT may be viewed as being composed of three functional domains, i.e. a carboxyl-terminal 50 kDa domain which mediates binding to the target neurons, a 50 kDa middle domain which assists or is responsible for internalisation of the BoNT, and an amino-terminal 50 kDa domain which functions as a zinc protease. Botulism can be prevented by the use of vacines. Botulism vacines currently available are generally formalin inactivated culture supernatants of *C. botulinium* types C and D grown in a corn steep liquor medium in dialysis tubing. Production of these vacines suffers from several drawbacks including instability of the *C. botulinium* vacine production strains, low levels of toxin production, damage of antigenic determinants during toxoiding and long production times. Large amounts of experimental animals are involved in quality control, which should be reduced if possible for ethical reasons. Working with highly toxic preparations and processing of the toxin for inactivation with formalin that can destroy the immunogenicity and protective ability of the antigen also form part of the current problems associated with current botulism vaccines.

According to one aspect of the invention, there is provided a non-toxic immunogenic derivative of *C. botulinum* type D neurotoxin or an immunogenic fragment thereof, said derivative or fragment carrying at least one mutation in its amino acid sequence, not found in wild-type D neurotoxins.

Non-toxic is defined as the intra peritoneal injection of 0.2 ml of the culture supernatant of a bacterial strain containing immunogenic derivatives of the *C. botulinum* type D neurotoxin in adult mice, not resulting in death for 10 days.

The derivative or immunogenic fragment thereof may carry a plurality of mutations in it's amino acid sequence, the mutations being selected from at least one of replacement mutations, substitution mutations, deletion mutations, insertion mutations, and inversion mutations.

According to another aspect of the invention, there is provided a non-toxic immunogenic derivative of *C. botulinum* type D BoNT which comprises a polypeptide having the deduced amino acid sequence of sequence ID No. 1 or a fragment, analog or derivative thereof.

According to a further aspect of the invention, there is provided a non-toxic immunogenic derivative of *C. botulinum* type D BoNT which comprises a polypeptide having the deduced amino acid sequence of sequence ID No. 2 (amino acids 1 to 399) or a fragment, analog or derivative thereof.

More specifically, the invention provides a non-toxic immunogenic derivative of *C. botulinum* type D BoNT which comprises a polypeptide having an amino acid sequence which is at least 75%, more preferably 85%, identical to an amino acid sequence selected from the group consisting of:

(i) amino acids 887 to 1285 of sequence ID No. 1, and

(ii) amino acids 1 to 399 of sequence ID No. 2.

An immunogenic fragment thereof is understood to be a fragment that, although not comprising the full length amino acid sequence of the derivative of the type D neurotoxin, still comprises regions of the derivative that are capable of inducing a protective immune response in the host animals.

A mutation is understood to be a change in the nucleic acid sequence of the derivative type D BoNT in comparison to the nucleic acid sequence of the wild-type type D BoNT.

As mentioned above, the mutation may be a replacement, substitution, deletion, insertion or inversion, or a combination thereof. A mutation can e.g. be such that one or more amino acids of the type D BoNT are replaced by other amino acids, with different characteristics.

Accordingly, the invention also relates to derivatives of type D BoNT according to the invention, wherein at least one mutation is a replacement mutation and/or wherein at least one mutation is a deletion or insertion.

When two or more mutations are made, combinations of replacement and deletion/insertion mutations are equally possible.

Non-toxic immunogenic derivatives of type D BoNT according to the invention may be made by introducing mutations in the gene encoding the type D BoNT. The mutated DNA fragments may then be cloned in a nucleotide sequence, such as a suitable expression plasmid and subsequently be expressed in a suitable host cell.

According to another embodiment of the invention, there is provided a nucleotide sequence comprising a mutated or recombinant DNA fragment that has as a characteristic that it encodes a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof according to the invention.

Accordingly, the invention provides a nucleic acid characterised by the nucleotide sequence of at least one of sequence ID No. 1 and sequence ID No. 2 (nucleotides 58 to 1254), or a fragment of said nucleotide.

In other words, the invention provides a nucleic acid comprising a nucleotide sequence which encodes a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof, said nucleotide sequence being selected from the group consisting of sequence ID No. 1, sequence ID No. 2 (nucleotides 58 to 1254), and a fragment of said nucleotide sequence.

More preferably, the invention comprises a nucleotide sequence comprising a polynucleotide having at least 75%, preferably 85%, identity to a member selected from the group consisting of:

(i) a polynucleotide of sequence ID No. 1;

(ii) the complement of (i);

(iii) a polynucleotide comprising nucleotides 58 to 1254 of sequence ID No. 2; and

(iv) the complement of (iii),

said polynucleotide encoding a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof.

More preferably, the invention comprises a nucleotide sequence comprising a polynucleotide having at least 75%, preferably 85%, identity to a member selected from the group consisting of:

(i) a polynucleotide of sequence ID No. 3; and

(ii) the complement of (i),

said polynucleotide encoding a genetically non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof.

The polynucleotide may be DNA or RNA. Preferably, the polynucleotide is plasmid DNA.

A suitable bacterial expression system for expressing the non-toxic immunogenic derivatives of type D BoNT according to the invention are Gram positive bacteria such as *Bacillus brevis*, *Bacillus subtilus* or Gram negative bacteria such as *Escherichia coli*. It is also envisaged that other expression systems may be used for expressing non-toxic immunogenic derivatives of type D BoNT. Other possible expression systems may be a suitable yeast expression system, for example *Pichia pastoris*.

In another embodiment of the invention, there is provided a Gram positive bacterial expression system, comprising a nucleotide sequence

according to the invention encoding a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof .

In a further embodiment of the invention, there is provided a Gram negative bacterial expression system comprising a nucleotide sequence according to the invention encoding a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof.

According to a further embodiment of the invention, there is provided a vaccine for protection against botulism caused by *C. botulinum* type D BoNT, said vaccine comprising a non-toxic immunogenic derivative of *C. botulinum* type D neurotoxin or an immunogenic fragment thereof according to the invention, and a physiologically acceptable carrier.

Such vaccines may be made by admixing an immunologically sufficient amount of a non-toxic immunogenic derivative or derivatives of *C. botulinum* type D BoNT according to the invention and a physiologically acceptable carrier.

An immunologically sufficient amount is understood to be the amount of non-toxic immunogenic *C. botulinum* type D BoNT derivative that is capable of inducing a protective immune response in a host animal.

Thus still another embodiment of the invention relates to vaccines for protection against *botulinum* caused by *C. botulinum* type D BoNT that comprises a non-toxic immunogenic derivative of *C. botulinum* type D BoNT according to the invention or an immunogenic fragment thereof, and a physiologically acceptable carrier.

Optionally, one or more compounds having adjuvant activity may be added to the vaccine, e.g. Alhydrogel, Alum or Saponin.

The vaccine may be administered to all hosts sensitive to *C. botulinum* type D BoNT, such as cattle, mules, sheep, goats, horses, birds, humans, etc.

It will be appreciated that an effective dosage of 1 ml subcutaneously for sheep and goats or 2 ml subcutaneously for cattle, mules and horses of the vaccine will be administered per animal. The dosage is administered once and may be repeated after a month or two.

According to another aspect of the invention, there is provided a method for preparing a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof according to the invention, which method comprises expressing in a suitable host cell, a nucleotide sequence according to the invention.

Non-toxic immunogenic derivatives of *C. botulinum* type D BoNT according to the invention may be made by replacing or modifying amino acids of the polypeptide or protein. The non-toxic immunogenic derivatives may also be prepared by introducing mutations in the gene or nucleic acid sequence encoding the *C. botulinum* type D BoNT. It will be appreciated that the non-toxic immunogenic derivatives may be prepared by suitable recombinant DNA techniques known in the art. The recombinant DNA or fragments thereof may then be cloned in a nucleotide sequence, such as a suitable expression vector, and subsequently be expressed.

According to a further aspect of the invention, there is provided a method for the preparation of a vaccine for combating *C. botulinum* type D BoNT poisoning, which method comprises admixing a non-toxic immunogenic derivative of *C. botulinum* type D BoNT according to the invention and a physiologically acceptable carrier.

The vaccine may be administered by a variety of suitable routes including internal, for example oral, nasal, or parenteral administration, for example by the intravenous, subcutaneous and intramuscular.

According to a further aspect of the invention, there is provided a vector which contains a polynucleotide of sequence ID No. 1 which

encodes a polypeptide having the deduced amino acid sequence of sequence ID No. 1.

According to another aspect of the invention, there is provided a vector which contains a polynucleotide of sequence ID No. 2 (nucleotides 58 to 1254) which encodes a polypeptide having the deduced amino acid sequence of sequence ID No. 2 (amino acids 1 to 399).

Accordingly, the invention provides a vector which includes a nucleotide sequence according to the invention which encodes a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof.

The invention also extends to a host cell genetically engineered with a vector as described herein.

The host cell may be any suitable host cell as a yeast or bacterium. Accordingly the host cell may be *B. subtilus, E. coli.* or *B. brevis*, e.g. *B. brevis* strain 47.

The vector may be any suitable vector known in the art, such as a suitable plasmid.

The invention also extends to a method for preparing a non-toxic immunogenic derivative of *C. botulinum* type D BoNT as herein described, which method comprises expressing in a Gram positive bacterium, a nucleotide sequence according to the invention encoding a non-toxic immunogenic derivative of *C. botulinum* type D BoNT.

The invention also provides a method for preparing a non-toxic immunogenic derivative of *C. botulinum* type D BoNT as herein described, which method comprises expressing in a Gram negative bacterium, a nucleotide sequence according to the invention encoding a non-toxic immunogenic derivative of *C. botulinum* type D BoNT.

The nucleotide sequence may be a polynucleotide of sequence ID No. 1 or sequence ID No. 2 (nucleotides 58 to 1254).

The nucleotide sequence may be expressed under the control of its native promoter or under the control of a heterologous promoter.

The Gram positive bacterium may be selected from the group consisting of *Bacillus brevis* and *Bacillus subtilus*.

The Gram negative bacterium may be *E. coli*.

According to yet another aspect of the invention, there is provided a process for producing cells capable of expressing a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof, said process comprising genetically engineering cells with a vector or plasmid as herein described.

According to a further aspect of the invention, there is provided a method of vaccinating an animal against *C. botulinum* type D BoNT, said method comprising administering an immunologically effective amount of a vaccine as herein described to the animal.

According to another aspect of the invention, there is provided a substance or composition for use in a method of vaccinating an animal against *C. botulinum* type D BoNT, said substance or composition comprising a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof as herein described, and said method comprising administering an immunologically effective amount of said substance or composition to said animal.

According to yet a further aspect of the invention, there is provided use of a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof as herein described in the

manufacture of a vaccine to vaccinate animals against *C. botulinum* type D BoNT poisoning.

The invention will now be described, by way of non-limiting example, with reference to the following Examples and Figures in which:

Figure 1 shows the structure of a suitable expression-secretion vector. The closed bar indicates the 5′ region of the MWP gene containing multiple promoters and a signal peptide-coding sequence. The open bar indicates a multiple cloning site (MCS). The DNA and amino acid sequences of these regions are shown in the upper part of the Figure. Vertical arrows along the top of the DNA sequence indicate transcription start sites 1-5. SD1 and SD2 are the ribosome-binding sites located upstream of the dual translation initiation sites. The signal peptide-coding sequence is underlined (reference 7). The broken line indicates the position of the nucleic acid sequence encoding a non-toxic immunogenic derivative of *C. botulinum* type D BoNT;

Figure 2 is a genetic map of a gene in accordance with the invention incorporated in a suitable vector for maintenance in *E. Coli*;

Figure 3 is a genetic map indicating sequencing coverage and sequence primer location. Included is a list of primers used and their sequences;

Figure 4 is an analysis of recombinant plasmids through comparative restriction enzyme digests. Lane 1 represents DNA molecular weight marker standards. Lanes 2 and 4 represent plasmid extractions from *B. brevis* transformants compared with lane 6, representing a PNU 211 plasmid profile. Hind III and Pst I restriction enzyme digests of PNU 211 recombinant plasmids (lanes 3 and 5) were compared with the same restriction enzyme digests of plasmid PNU 211 before ligation with the gene fragment in accordance with the invention and GeneOp-ABC plasmid carrying the gene fragment according to the invention (lanes 7 and 8 respectively). The arrows indicate the respective sizes of the DNA fragments in base pairs (bp). The arrows indicating the DNA fragments of 1207 bp, represent the genes according to the invention as digested from the recombinant PNU 211 plasmids and the GeneOp-ABC plasmid;

Figure 5 is a PAGE electrophoretogram of *B. brevis* culture supernatant demonstrating secretion of recombinant heterologous COOH-heavy chain fragment of the *C. botulinum* type D neurotoxin into modified PY medium by *B. brevis* 115 as determined by western blot analysis (Fig 13). Lane 1 represents the protein profile of the culture supernatant of a

B. brevis strain transformed with PNU 211 without the gene fragment according to the invention. Protein profiles, of samples of culture supernatant of *B. brevis* 115 (transformed with PNU 211 ligated with the gene according to the invention) prepared under non-reducing and reducing conditions, are presented in lanes 2 and 3 respectively. The arrow indicates the position of the recombinant protein;

Figure 6 is a Western blot analysis of a PAGE protein profile of *B. brevis* culture supernatants demonstrating secretion of recombinant heterologous COOH-heavy chain fragment into modified PY-medium by *B brevis* 115. The recombinant fragment protein band was detected by treating the filter with polyclonal antibodies raised against a crude extract of the native neurotoxin of *C. botulinum* type D. Lane 1 represents the protein profile of a crude extract of the native neurotoxin protein complex of *C. botulinum* type D. Protein profiles of samples of culture supernatant of *B. brevis* 115, prepared under non-reducing and reducing conditions, are presented in lanes 2 and 3, respectively. Lane 4 represents the protein profile of the culture supernatant of a *B. brevis* strain transformed with PNU 211 without the gene fragment according to the invention. The arrow indicates the position of the recombinant fragment protein according to the invention; and

Figure 7 is a growth curve of a fermentation culture of *B. brevis* strain 115 in modified PY-medium.

Sequence ID No. 1 is a nucleotide sequence of a synthetic gene encoding a non-toxic immunogenic derivative of *C. botulinum* type D toxin (Sequence ID No. 1 -nucleotide and amino acid sequences).

Sequence ID No. 2 shows a nucleic acid sequence of a gene encoding a non-toxic immunogenic derivative of type D BoNT according to the invention (sequence ID No. 2 - nucleotide and amino acid sequences) including portions of a suitable plasmid which are immediately upstream and downstream of the gene. Included is a list of restriction sites and amino acid composition.

Example

A novel nucleotide sequence or gene according to the invention encoding a non-toxic immunogenic derivative of *C. botulinum* type D toxin (BoNT) was created using an amino acid sequence of *C. botulinum* type D strain South Africa (Dsa) (Reference 3) to start from. The nucleotide sequence of amino acids Nos. 887 - 1285 of the heavy chain COOH(Hc) neurotoxin fragment was recorded (Sequence ID Nos 1 and 2). The nucleotide sequence or gene was redesigned to have the optimal codons for expression in *B. brevis* by making use of the *B. brevis* codon table.

Materials and methods

Bacterial strains and vectors.

An *Escherichia coli* strain JM 109 (Reference 3) was used for the amplification of the gene according to the invention. *Bacillus brevis* strain 47-5Q (JMC no. 8970) was obtained from the Japanese Collection of Microorganisms, The Institute of Physical and Chemical Research, Wako-shi, Saitama, Japan. Plasmid PNU 211 was obtained from S Udaka, Department of Applied Biological Sciences, Nagoya University, Japan.. Clones were selected by growth on LB agar plates supplemented with 50 μg/ml ampicillin. Positive clones were cultured in LB-broth supplemented with 50 μ g/ml ampicillin (Reference 5). Plasmid extractions were

performed according to the method of Reference 19. Plasmids were digested with restriction enzymes Pst I and Hind III and analyzed on a 1% agarose gel to confirm the presence of the gene fragment according to the invention (Reference 19). The gene fragment according to the invention was digested with Pst I and Hind III, and the gene fragment ligated with plasmid PNU 211 (S Udaka, Department of Applied Biological Sciences, Nagoya University, Japan - Reference 27). *B. brevis* strain 47 - 5q (deposited under No. JMC 8970 Japan Collection of Microorganisms, The Institute of Physical and Chemical Research, Waka-shi, Saitama, Japan, 350-01) was cultured in T2U-medium (Udaka and Yamagata 1993 - reference 28). Transformations were performed according to the method of Reference 2. The *B. brevis* transformants were cultured in T2U-medium supplemented with 10 μg/ml erythromycin and plasmid extractions performed according to the method of Reference 1. Plasmids were analyzed for the correct insertion by restriction enzyme Pst I and Hind III digests and analyzed on a 1% agarose gel. Transformants were screened for expression in T2U-medium, PM-medium (Reference 28), 5YC-medium (Reference 2) and PY-medium (Reference 15) through colony blot analysis using polyclonal antibodies raised against the native neurotoxin of *C. botulinum* type D.

Enzymes and reagents.

Calibration Proteins for SDS gel electrophoresis were from Boehringer Mannheim. ECL Western blotting detection reagents and ECL protein

molecular weight markers were obtained from Amersham International. Shrimp alkaline phosphatase was obtained from Boehringer Mannheim. T4 DNA Ligase and restriction enzymes were obtained from Promega. HRP conjugated Protein G was obtained from Zymed Laboratories Inc. Hybond-C nitrocellulose membranes, supplied by Amersham International, were used for colony blot analysis. PVDP membranes used for Western blots were obtained from Microcept. Onderstepoort Biological Products (OBP) Onderstepoort, South Africa supplied polyclonal antibodies against the native neurotoxin of *C. botulinum* type D. Standardized (u/ml) *C. botulinum* type D neurotoxin was also obtained from OBP, Onderstepoort, South Africa. DAB substrate kit was obtained from Zymed Laboratories Inc. Difco supplied Proteose Peptone. Fluka was the supplier of Polyethylene glycol 6000.

Synthetic gene or nucleotide sequence in accordance with the invention.

A synthetic gene or nucleotide sequence according to the invention was created using the amino acid sequence of *C. botulinum* type D strain South Africa (Dsa) Reference 14 as a starting point. The nucleotide sequence of amino acids nos. 887 to 1285 of the heavy chain COOH (Hc) neurotoxin segment was recoded. The gene according to the invention was designed to include optimal codons for expression in *Bacillus brevis* by making use of the *Bacillus brevis* codon table. A codon optimization program was used for construction, eliminating any rare (4 to 8 in a thousand) to very rare (4

or less in a thousand) codons and then to use representative numbers of the remaining codons. Restriction enzyme recognition sites for Pst I and Hind III were included for cloning purposes.

The gene was synthesized and cloned into a suitable vector [GeneOp vector (Operon technologies)] as shown in Figures 2 and 3. The vector carrying the synthetic gene fragment according to the invention was transformed into an *Escherichia coli* strain Jm 109 according to the method of Reference 19.

Antisera.

Antiserum used for immunoblots was prepared by hyper-immunization of horses against *C. botulinum* serotype D neurotoxin (OBP). Antibodies present in the serum, cross-reacting with *Bacillus brevis* cell proteins, were removed through adsorption with *B. brevis* acetone powder (Reference 5).

***Bacillus brevis* recombinants.**

As mentioned above, the GeneOp vector carrying the synthetic gene fragment (GeneOp-ABC) was transformed into *Escherichia coli* strain JM 109 (Reference 19). Clones were selected by growth on LB agar plates supplemented with 50 μg/ml ampicillin. Positive clones were cultured in LB-broth supplemented with 50 μg/ml ampicillin (Reference 19). Plasmid extractions were performed according to the method of Reference 19. (1989). Plasmids were digested with restriction enzymes Pst I and Hind III

and analyzed on a 1 % agarose gel to confirm the presence of the gene fragment (Reference 19). All restriction enzyme digests were performed according to the instructions of the manufacturer. *Bacillus brevis* strain 47-5Q (JMC no. 8970) was transformed with PNU 211 according to the method of Reference 28. Clones containing PNU 211 were selected by growth on T2U plates supplemented with 10 μg/ml erythromycin (Reference 28). *B. brevis* containing the PNU 211 plasmid was cultured in T2U-medium supplemented with 10 μg/ml erythromycin. Plasmids were extracted according to the method of Reference 2 and analyzed on a 1% agarose gel. GeneOp-ABC was digested with Pst I and Hind III, and dephosphorilated using Shrimp alkaline phosphatase according to the instructions of the manufacturer. The GeneOp-ABC preparation (5 μg) was ligated with 5 μg PNU 211, digested with Pst I and Hind III, using T4 DNA ligase according to the instructions of the manufacturer. Transformation of *B. brevis* with the ligation mixture was performed according to the method of Reference 28. Control transformations with undigested PNU 211 were included. Clones were selected by growth on T2U plates supplemented with 10 μg/ml erythromycin. PNU 211 transformants with the gene fragment insert were screened by immuno colony blot analysis. Identified transformants that produced a heterologous protein were picked from the plates and grown on fresh plates. The *B. brevis* transformants were cultured in T2U-medium supplemented with 10 μg/ml erythromycin and plasmid extractions performed according to the method of Reference

2. Recombinant plasmids were analyzed for the correct insert by restriction enzyme Pst I and Hind III digests in comparison with Pst I and Hind III digested GeneOP-ABC plasmid material on a 1% agarose gel.

Immuno colony analysis.

Plates of transformants were covered with sterile nitrocellulose membranes for 1 to 3 hours. After removal the colonies transferred to the membranes were lysed by treating the membranes with lysing buffer (50 mM Glucose, 10 mM EDTA, 25 mM Tris, 0.5 % lysozyme, pH 8.0) for 1 hr at 37 °C. Membranes were transferred in a Trans-Blot SD semi-dry electrophoretic transfer cell (Bio-Rad) with colony side facing for 30 min at 25 V (Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell Intruction Manual). After transfer, the membranes were washed 2 x for 15 min in TBS buffer (Reference 19) at room temperature. The membranes were then incubated in TBS containing 10 μg/ml Dnase I at room temperature for at least 10 min with gentle shaking on an orbital shaker. The membranes were placed in TBS buffer supplemented with 10 % fat free milk powder for 1 hr or overnight at 4 °C. Adsorbed Onderstepoort *C. botulinum* antiserum type D (1000 units/ml) was diluted 1: 200 in TBS (1% milk powder) and used as primary antibody solution. Membranes were incubated with primary antibody solution for 1 h at room temperature on an orbital shaker. The membranes were then washed 3 times for 5 min at room temperature. Finally, the membranes were incubated for 1 hr in Horseradish peroxidase (HRP) conjugated Protein G (Zymed), diluted 1: 3000 in TBS (1% milk

powder), and afterwards washed 3 times for 5 min in TBS. A positive reaction was detected by using a 3,3=-Diaminobenzidine tetrahydrochloride (DAB) substrate kit (Zymed) for HRP according to the instructions of the manufacturer.

Preservation of cultures.

B. brevis strains were cultured in T2U medium at 37 °C for 24 h. Cultures were stored in T2U medium containing 25 % (v/v) glycerol at -70 °C.

Protein expression and secretion.

B. brevis transformants carrying the gene fragment according to the invention were cultured in PY medium (Reference 15), modified as follows: [Proteose peptone (20g), yeast extract (1.5g), glucose (15g), and uracil (0.5g)/liter] supplemented with mineral mixture (0.01835% $FeSO_4.7H_2O$, 0.0008 % $MnSO_4.7H_2O$, 0.0001% $ZnSO_4.7H_2O$), 0.1% $MgSO_4.7H_2O$ and 20 μg/ml erythromycin. Modified PY medium (100 ml) was inoculated with 1 ml of a glycerol store culture and incubated for 4 days at 30 °C as a shake culture. Culture were harvested and centrifuged to remove bacterial cells. Protein profiles of the culture supernatant were prepared and compared with the negative control strain (*B. brevis* transformed with PNU 211) through SDS-PAGE electrophoresis, PAGE electrophoresis and Western blot analysis.

PAGE electrophoresis.

PAGE electrophoresis (Bio-Rad Mini Protean II) on a 5% gel were performed as described by Hames and Rickwood (1990) using the non-

dissociating high pH discontinuous system. Sample preparation occurred under both reducing and non-reducing conditions. Samples were diluted 1:4 with stacking gel buffer supplemented with 40 % sucrose and 0.008 % Bromophenol blue. To create reducing conditions when necessary, samples were diluted 1:4 with stacking gel buffer supplemented with 40 % sucrose, 0.008 % Bromophenol blue, 8 % SDS and 2 % Dithiothreitol and boiled for 5 min. Gels were fixed for 20 min in fixing solution (400 ml ethanol, 100 ml glacial acetic acid, 500 ml distilled water) and then exposed to destain solution (300 ml ethanol, 80 ml glacial acetic acid, 620 ml distilled water) for 2 min. Gels were stained for 10 min in staining solution (0.1 % Coomassie blue in destain solution) and then destained in destain solution.

Western blot analysis

Proteins were transferred from PAGE gels to PVDF membranes using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (BioRad Instruction manual). Towbin transfer buffer (BioRad Instruction manual) was modified by increasing the methanol concentration in the buffer from 200 ml to 220 ml. The Western blot analysis was completed following the method as described for the Immuno colony analysis. Onderstepoort *C. botulinum* antiserum type D (1000 units/ml) was, after adsorption, diluted 1:200 and used as primary antibody. Horseradish peroxidase (HRP) conjugated Protein G was diluted 1: 3000 and used as secondary antibody. The ECL Western blotting detection reagent kit (Amersham International) was used following

the Instruction Manual of the manufactures of the ECL kit. Positive reactions were visualized using the Lumi-Imager (Boehringer Mannheim) and LumiAnalyst Image Analysis software program (Boehringer Mannheim). The concentration of the recombinant fragment protein band, identified by Western blot analysis, was calculated according to the relative % of the band to the total protein concentration loaded per sample using the LumiAnalyst Image Analysis software program (Boehringer Mannheim).

Fermenter production

A non-toxic neurotoxin fragment according to the invention was produced in a B. Braun Biotech International fermenter in 10 liter modified PY-medium supplemented with 0.05 % Tween 20. T2U medium (100 ml) was inoculated with 1 ml of a glycerol store culture and incubated for 24 h at 37 °C as a shake culture. The 24-h T2U culture was then used to inoculate each of 5 flasks containing 100 ml modified PY-medium with 20-ml culture. The modified PY shake cultures were incubated for 24 hr at 37 °C and then used as inoculum for the 10 liter quantity fermenter system. After completion of the fermentor run at day 4, the contents were harvested and centrifuged to remove the bacterial cells. The culture supernatant was analysed for the presence of the protein fragment according to the invention with dot-blot hybridization based on the method as described for the Immuno colony assay. Samples were also withdrawn at daily intervals during the fermenter run and culture supernatant analyzed

with dot-blot hybridization to monitor the protein expression and secretion of the protein fragment during the process.

Vaccination of animals.

The culture supernatant was mixed in a 1: 1 ratio with 50 % Aluminium Hydroxide adjuvant (OBP) and used as a vaccine to immunize groups of 5 healthy outbred BalbC (of the colony maintained at OBP) mice, each weighing 18 g to 20 g. The mice were each injected subcutaneously with 0.2 ml. After 21 days, two groups of 5 immunized mice and 10 control mice each, were challenged with OBP *C. botulinum* type D standard neurotoxin. One group of immunized mice and one group control mice were injected intraperitoneally into each of the mice with 0. 2 ml of toxin diluted in saline to give a final concentration of 0.05 U. The other group of immunized and control mice were each challenged with 0.03 U toxin. The mice were observed for 24 h for survival, signs of botulism or death.

RESULTS AND DISCUSSION

Synthetic gene composition.

The nucleotide sequence of the synthetic gene fragment according to the invention with restriction enzyme sites is shown in Figures 3 and Sequence ID Nos 1 and 2. The size of the gene is 1207 bp and the resulting protein after expression 399 amino acids long. The percentage of rare codons used was 0.3 %. The %GC content is 34.6 %, compared to the %GC of

B. brevis of 42.7 to 47 % (Reference 27) and to that of *C. botulinum* of 26 to 28 %.

***Bacillus brevis* recombinants.**

The construction of the PNU 211 vector is shown in Fig. 1. At the Pst I site, the gene fragment according to the invention was fused to the 5= region of the MWP gene. Restriction enzyme digests of the GeneOp vector carrying the gene fragment before and after amplification in *E. coli*, control plasmid PNU 211 and recombinant PNU211 plasmids isolated from the transformants were compared (Fig 4). A 1.2 Kbp fragment could be excised from the plasmids with Pst I and Hind III. This demonstrated the presence of an insert in the transformants with a size comparable with that of the synthetic gene according to the invention.

Protein expression and secretion.

Immunoreactive transformant colonies were identified. One of these transformants (*B. brevis* 115) was cultured on modified PY-medium and the culture supernatant subjected to PAGE electrophoresis and Western blot analysis to demonstrate protein expression and secretion. As expressed in *B. brevis*, the gene according to the invention produces and secretes a polypeptide into the culture medium of modified PY-medium (Fig. 5) that reacts in Western blots (Fig. 6) with antisera to serotype D botulinum toxin.

Fermenter production

Process constraints due to genetic instability of genetically manipulated or altered microorganism is more significant on commercial scale than laboratory scale. Production started with a batch-growth phase in the production medium, used as a 20 % inoculum for 10 liter medium in the fermenter. Figure 7 represents the growth curve of a typical fermenter run. Temperature was maintained at 30 °C throughout the run. The pH values were monitored and used as a growth indicator. Bacterial growth reached the stationary phase in approximately 4 days.

The fermenter was set at maximum value for oxygen saturation. As bacterial growth entered the exponential growth phase the PO2 value decreased dramatically and only started to recover to the initial value as the growth reached the stationary phase. This demonstrates the dependency on oxygen for maximal growth of *B. brevis*. Maximum values for production and secretion of the protein according to the invention into the culture medium were obtained after 4 days when growth entered the stationary phase. Higher yields of the secreted protein according to the invention were detected in the culture fluid using the fermenter system, compared with a batch culture. This might be attributed to more sufficient continuous supply of oxygen in the fermenter, especially after the stationary phase of growth is reached and the bacteria starts to shed the cell wall proteins into the medium, than in a batch culture. Extracellular

production of 1g/liter of the protein according to the invention was obtained using the fermenter system.

Vaccination of animals.

To obtain an indication of the protective antigenicity of the protein or polypeptide according to the invention in the formulation of a vaccine against *C. botulinum* type D neurotoxin, a crude culture supernatant was used to vaccinate mice. Mice challenge studies with 0.03 U and 0.05 U toxin, 21 days after vaccination, resulted in death of all the control mice in 24 h. In contrast, 60 % and 20 % mice vaccinated with the culture supernatant still survived for at least 24 h post challenging with 0.03 U and 0.05 U, respectively. The culture supernantant appeared to have no detrimental effects on the mice.

Advantages of the invention are that it provides a relatively simple, relatively inexpensive method of producing non-toxic immunogenic derivatives of *C. botulinum* type D BoNT and vaccines for combating *C. botulinum* type D BoNT. This method of vaccine production would involve fermenter technology, a unique method for botulism type D vaccine production compared to the current analysis production method. The vaccines can be produced or manufactured relatively quickly and the vaccine is non-toxic *per se*. Since the product of the fermentation process is non-toxic, the production procedures result in little or no health risk to production personnel.

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CLAIMS

1. A non-toxic immunogenic derivative of *C. botulinum* type D neurotoxin or an immunogenic fragment thereof, said derivative or fragment carrying at least one mutation in its amino acid sequence, not found in wild-type D neurotoxins.

2. A derivative as claimed in Claim 1, in which said derivative or immunogenic fragment thereof carries a plurality of mutations in its amino acid sequence, the mutations being selected from at least one of replacement mutations, substitution mutations, deletion mutations, insertion mutations, and inversion mutations.

3. A non-toxic immunogenic derivative of *C. botulinum* type D BoNT which comprises a polypeptide having the deduced amino acid sequence of sequence ID No. 1 or a fragment, analog or derivative thereof.

4. A non-toxic immunogenic derivative of *C. botulinum* type D BoNT which comprises a polypeptide having the deduced amino acid sequence of sequence ID No. 2 (amino acids 1 to 399) or a fragment, analog or derivative thereof.

5. A non-toxic immunogenic derivative of *C. botulinum* type D BoNT which comprises a polypeptide having an amino acid sequence which is at least 75 % identical to an amino acid sequence selected from the group consisting of:

(i) amino acids 887 to 1285 of sequence ID No. 1, and

(ii) amino acids 1 to 399 of sequence ID No. 2.

6. A nucleotide sequence comprising a recombinant DNA fragment characterised in that said DNA fragment encodes a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof according to any one of claims 1 to 5.

7. A nucleotide sequence comprising a mutated DNA fragment, characterised in that said DNA fragment encodes a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof according to any one of claims 1 to 5.

8. A nucleic acid comprising a nucleotide sequence which encodes a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof, said nucleotide sequence being selected from the group consisting of sequence ID No. 1, sequence

ID No. 2 (nucleotides 58 to 1254), and a fragment of said nucleotide sequence.

9. A nucleotide sequence comprising a polynucleotide having at least 75 % identity to a member selected from the group consisting of:

(i) a polynucleotide of sequence ID No. 1;

(ii) the complement of (i);

(iii) a polynucleotide comprising nucleotides 58 to 1254 of sequence ID No. 2; and

(iv) the complement of (iii),

said polynucleotide encoding a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof.

10. Expression system based upon a Gram negative bacterium comprising a nucleotide sequence according to any one of claims 6 to 9 encoding a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof.

11. Expression system based upon a Gram positive bacterium, said Gram positive bacterium not being *C. botulinum*, comprising a nucleotide sequence according to any one of claims 6 to 9 encoding a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof.

12. A vaccine for protection against botulism caused by *C. botulinum* type D BoNT, said vaccine comprising a non-toxic immunogenic derivative of *C. botulinum* type D neurotoxin or an immunogenic fragment thereof according to any one of claims 1 to 5, and a physiologically acceptable carrier.

13. A method for preparing a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof, which method comprises expressing in a host cell, a nucleotide sequence as claimed in any one of claims 6 to 9.

14. A vector which includes a nucleotide sequence according to any one of claims 6 to 9.

15. A host cell genetically engineered with a vector as claimed in claim 14.

16. A process for producing cells capable of expressing a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof, said process comprising genetically engineering cells with a vector according to claim 14.

17. A substance or composition for use in a method of vaccinating an animal against *C. botulinum* type D BoNT, said substance or composition comprising a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof as claimed in any one of claims 1 to 5, and said method comprising administering an immunologically effective amount of said substance or composition to said animal.

18. Use of a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof as claimed in any one of claims 1 to 5 in the manufacture of a vaccine to vaccinate animals against *C. botulinum* type D BoNT poisoning.

19. A method of vaccinating an animal against *C. botulinum* type D BoNT, said method comprising administering an immunologically effective amount of a vaccine as claimed in claim 12 to said animal.

20. A derivative of *C. botulinum* type D BoNT as claimed in any one of claims 1 to 5, substantially as herein described and illustrated.

21. A nucleotide sequence as claimed in any one of claims 6, 7 and 9, substantially as herein described and illustrated.

22. A nucleic acid as claimed in claim 8, substantially as herein described and illustrated.

23. An expression system as claimed in claim 10 or claim 11, substantially as herein described and illustrated.

24. A vaccine as claimed in claim 12, substantially as herein described and illustrated.

25. A method as claimed in claim 13, substantially as herein described and illustrated.

26. A vector as claimed in claim 14, substantially as herein described and illustrated.

27. A host cell as claimed in claim 15, substantially as herein described and illustrated.

28. A process as claimed in claim 16, substantially as herein described and illustrated.

29. A substance or composition for use in a method of vaccination as claimed in claim 17, substantially as herein described and illustrated.

30. Use as claimed in claim 18, substantially as herein described and illustrated.

31. A method as claimed in claim 19, substantially as herein described and illustrated.

32. A new derivative of *C. botulinum* type D BoNT, a new nucleotide sequence, a new nucleic acid, a new expression system, a new vaccine, a new method for preparing a compound, a new vector, a new host cell, a new process for producing cells capable of expressing a compound, a substance or composition for a new use in a method of vaccination, a new use of a non-toxic immunogenic derivative of *C. botulinum* type D BoNT or an immunogenic fragment thereof as defined in any one of claims 1 to 5, or a new method of vaccinating an animal, substantially as herein described.

```
                                                                    60
   Mf1I
ATCAGATCCGCTATCCTGTCTTACAACTTGGCTGTTGTAAACTTTGAAAATGCATTAGGA
                                                                   120
AATTAACCTAATTCAAGCAAGATTATGAGGTTTTGAACCAAATTGGAAAAAGGTTCAGTC
                                                  1↓               180
GTGACACCCCGCCATATGTCCCCTATAATACGGATTGTGGCGGATGTCACTTCGTACATA
                                                                   240
ATGGACAGGTGAATAACGAACCACGAAAAAAACTTTAAATTTTTTTCGAAGGCGCCGCAA
                                              2↓                   300
CTTTTGATTCGCTCAGGCGTTTAATAGGATGTCACACGAAAAACGGGGAATTGTGTAAAA
          EcoRI                    SpeI     3↓                    360
AAGATTCACGAATTCTAGCAGTTGTGTTACACTAGTGATTGTTGCATTTTACACAATACT
                                  4↓          5↓        SD1        420
GAATATACTAGAGATTTTTAACACAAAAACCGAGGCTTTCCTGCGAAAGGAGGTGACACG
                                                                   480
CGCTTGCAGGATTCGGGCTTTAAAAAGAAAGATAGATTAACAACAAATATTCCCCAAGAA
  fMetGlnAspSerGlyPheLysLysLysAspArgLeuThrThrAsnIleProGlnGlu
                                                  HpaI             540
                    SD2
CAATTTGTTTATACTAGAGGAGGAGAACACAAGGTTATGAAAAAGGTCCTTAACAGTGTA
GlnPheValTyrThrArgGlyGlyGlullisLysValMetLysLysValVnlAsnSerVal
                                  NcoI          PstI
      ApaLI
TTGGCTAGTGCACTCGCACTTACTGTTGCTCCCATGGCTTTCGCTGCAG==========
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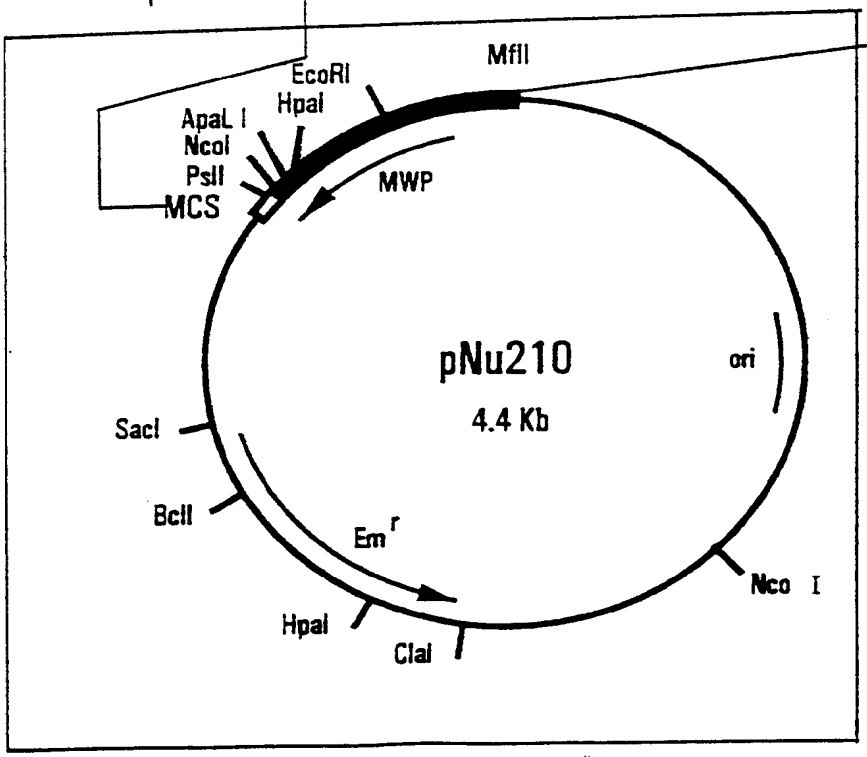

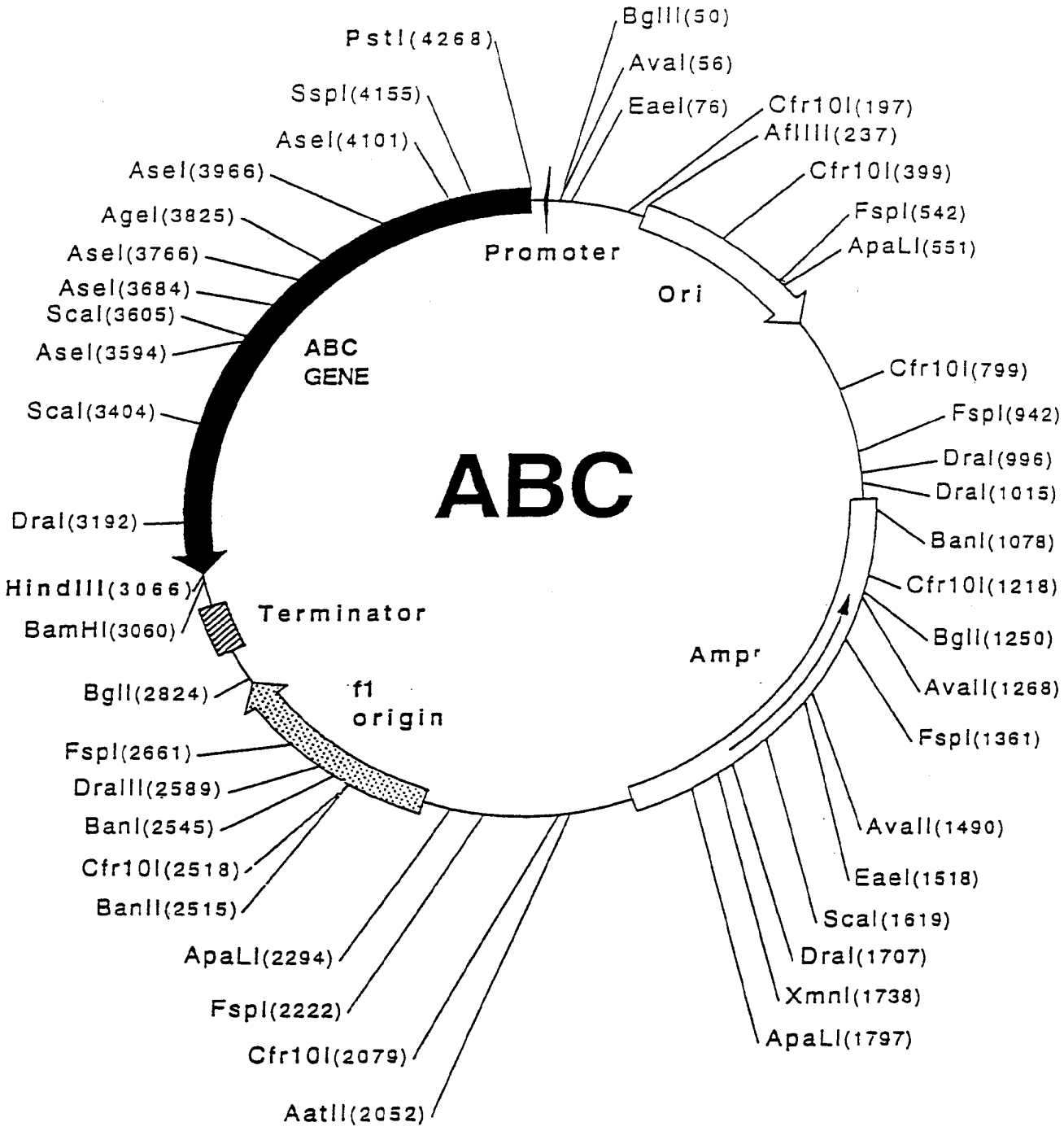
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Cfr10I(799)
FspI(942)
DraI(996)
DraI(1015)
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BglI(1250)
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EaeI(1518)
ScaI(1619)
DraI(1707)
XmnI(1738)
ApaLI(1797)
AatII(2052)
Cfr10I(2079)
FspI(2222)
ApaLI(2294)
BanII(2515)
Cfr10I(2518)
BanI(2545)
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FspI(2661)
BglI(2824)
BamHI(3060)
HindIII(3066)
DraI(3192)
ScaI(3404)
AseI(3594)
ScaI(3605)
AseI(3684)
AseI(3766)
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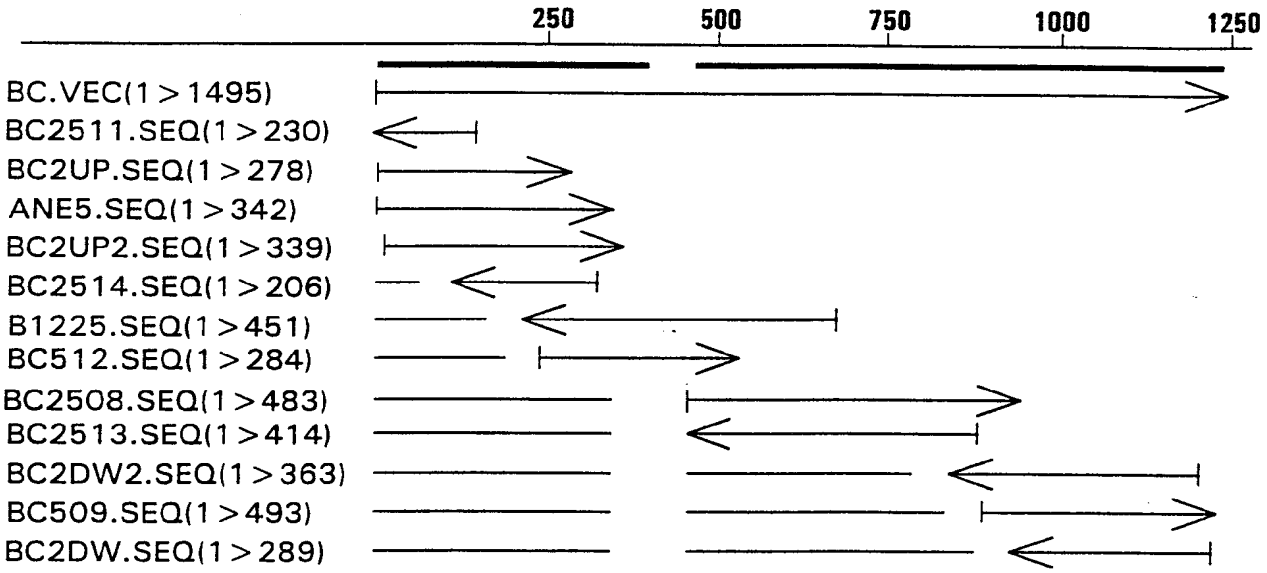
PstI(4268)

FIG. 2

FIG. 3A



Name of Sequencing Run	Name on Sequencing Chromatagram
ABC2UP, LANE5, and ABCUP2	ABC 1 + 2 (2) UP and ABC(1 + 2) 2 UP
ABC2DW2 and ABC2DW	ABC(1 + 2) 2DW and ABC1 + 2 (2) DW
ABC2508	ABC(1 + 2) 508
ABC509	ABC(1 + 2) 509
AB1225	ABC(1 + 2) 2.510
ABC2511	ABC1 + 2 (2) 511
ABC512	ABC1 + 2 (2) 512
ABC2513	ABC1 + 2 (2) 513
ABC2514	ABC (2) 514

Primer Sequence

5′ - CGG GAG ATC TCG ATC CCG CGA - 3′
5′ - GGC TTT GTT AGC AGC CGG ATC - 3′
5′ - CCA TTA CCA CAA ACA TGA TGG G - 3′
5′ - GAG TTC GTG GTG AGA ACG TGC - 3′
5′ - GTT GAA GAC GAT ACC GTT ACC C - 3′
5′ - CGC TGT CGA TGA TGG TAT AAC C - 3′
5′ - ATA CCA TCA TCG ACA GCG - 3′
5′ - GAT CTA ATG CAC CCA ATG G - 3′
5′ - GGT GTC GAT TAG CTT TCC G - 3′

FIG. 3B

Restriction Sites in GeneOp

ENZYME	SEQUENCE	#	LOCATIONS
Aat II	GACGTC	1	2048
Afl III	ACPuPyGT	1	237
Ahd I	GACNNNNNGTC	1	1125
Alw 26 I	GTCTC	5	12 1197 1962 2115 2168
*Alw*N I	CAGNNNCTG	1	648
*Apa*L I	GTGCAC	3	551 1797 2294
Apo I	PuAATTPy	1	2780
Ase I	ATTAAT	3	32 66 1301
Ava I	CPyCGPuG	1	56
Ava II	GG(A,T)CC	2	1268 1490
*Bam*H I	GGATCC	1	3060
Ban I	GGPyPuCC	2	1078 2545
Ban II	GPuGCPyC	1	2511
Bfa I	CTAG	6	732 985 1320 2433 2954 3071
Bgl I	GCCNNNNNGGC	2	1244 2818
Bgl II	AGATCT	1	50
Blp I	GCTNAGC	1	2963
Bpm I	CTGGAG	1	1215
Bsa I	GGTCTC	2	11 1197
*Bsa*A I	PyACGTPu	1	2584
*Bsa*B I	GATNNNNATC	1	45
*Bsa*H I	GPuCGPyC	2	1666 2048
*Bsa*J I	CCNNGG	3	56 397 2941
*Bsa*W I	(A,T)CCGG(A,T)	4	443 590 1421 2886
*Bsi*E I	CGPuPyCG	6	150 574 1497 1646 2552 2849
*Bsi*HKA I	G(A,T)GC(A,T)C	4	551 1712 1797 2294
Bsl I	CCNNNNNNNGG	9	79 253 271 437 716 2164 2361 2687 2928
*Bsm*B I	CGTCTC	2	2114 2168
Bsp 1286 I	G(G,A,T)GC(C,A,T)C	5	551 1712 1797 2294 2511
*Bsp*E I	TCCGGA	1	2886
*Bsp*H I	TCATGA	3	957 1965 2070
Bsr I	ACTGGN	9	640 653 770 1176 1294 1337 1601 1776 2670
*Bsr*B I	GAGCGG	3	168 1969 2440
*Bsr*D I	GCAATGNN	2	1184 1366
*Bsr*F I	PuCCGGPy	2	1210 2481
*Bss*S I	CTCGTG	3	410 1794 2101
*Bst*N I	CC(A,T)GG	4	264 385 398 2878
*Bst*Y I	PuGATCPy	7	50 878 889 975 987 1755 1772
Csp 6 I	GTAC	2	1609 2285
Dde I	CTNAG	8	512 921 1087 1627 2053 2288 2964 2985
Dra I	TTTAAA	3	994 1013 1705

FIG. 3C

Restriction Sites in GeneOp

Dra III	CACNNNGTG	1	2584								
Drd I	GACNNNNNNGTC	3	339	2208	2627						
Eae I	PyGGCCPu	2	76	1518							
Ear I	CTCTTC	3	115	1919	2863						
Eco 0109 I	PuGGNCCPy	2	2105	2936							
Eco 57 I	CTTCAG	2	764	1812							
Fau I	GCGGG	7	41	86	2231	2241	2385	2439	2857		
Fok I	GGATG	4	1110	1291	1578	2194					
Fsp I	TGCGCA	2	1350	2829							
Hae II	PuGCGCPy	4	111	481	2427	2435					
Hga I	GACGC	5	339	917	1667	2225	2355				
Hinf I	GANTC	8	25	72	137	212	608	1125	2634	2656	
Hph I	GGTGA	9	981	1208	1604	1830	1845	2129	2138	2574	3034
Mbo II	GAAGA	8	116	887	978	1733	1811	1920	2455	2864	
Msl I	CAPyNNNNPuTC	3	1378	1537	1896						
*Msp*A1 I	C(A,C)GC(G,T)G	5	577	822	1763	2229	2966				
Nci I	CC(C,G)GG	8	56	57	616	1312	1641	1663	2164	2199	
*Ngo*M I	GCCGGC	1	2481								
Nla III	CATG	9	238	958	1449	1459	1537	1573	1966	2071	2155
Nsp I	GCATGT	2	237	2154							
Ple I	GAGTC	6	25	137	608	1125	2634	2656			
Pvu I	CGATCG	2	1497	2849							
Sap I	GCTCTTC	1	114								
Sau 96 I	GGNCC	8	1172	1251	1268	1490	2106	2592	2859	2937	
Sca I	AGTACT	1	1608								
Sfc I	CTPuPyAG	6	18	502	693	1371	2363	3041			
Ssp I	AATATT	3	1932	2768	2792						
*Sta*N I	GCATC	8	325	1377	1587	1817	2195	2270	2325	2346	
Sty I	CC(A,T)(A,T)GG	1	2941								
Tai I	ACGT	9	940	1356	1729	2049	2475	2585	2628	2640	2799
Taq I	TCGA	5	47	300	337	1781	2551				
Tfi I	GA(A,T)TC	2	72	212							
Tsp 45 I	GT(C,G)AC	5	1387	1598	2174	2409	3035				
Tsp 509 I	AATT	6	35	998	1304	1559	2781	2807			
*Tsp*R I	CAGTG	8	133	639	652	923	1072	1177	1524	1551	
Xba I	TCTAGA	1	3070								
Xma I	CCCGGG	1	56								
Xmn I	GAANNNNTTC	1	1725								

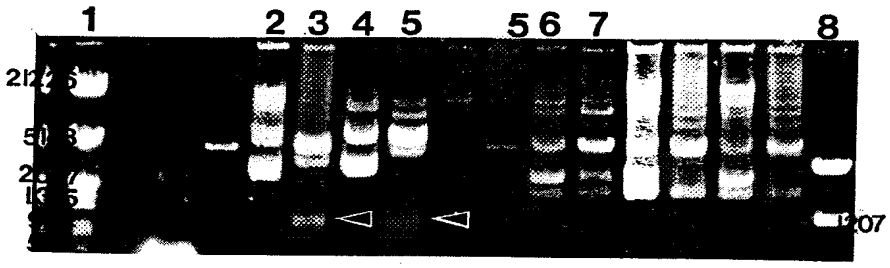


FIG. 4

FIG. 5

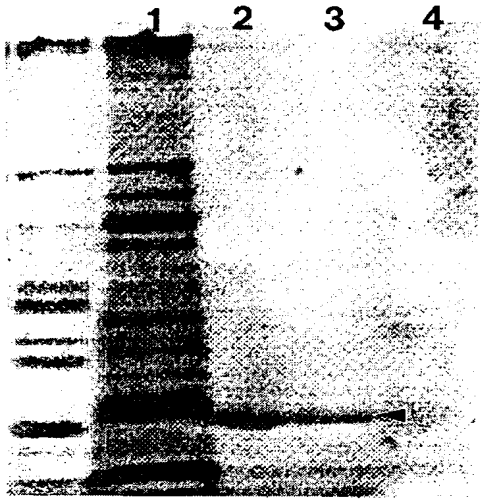


FIG. 6

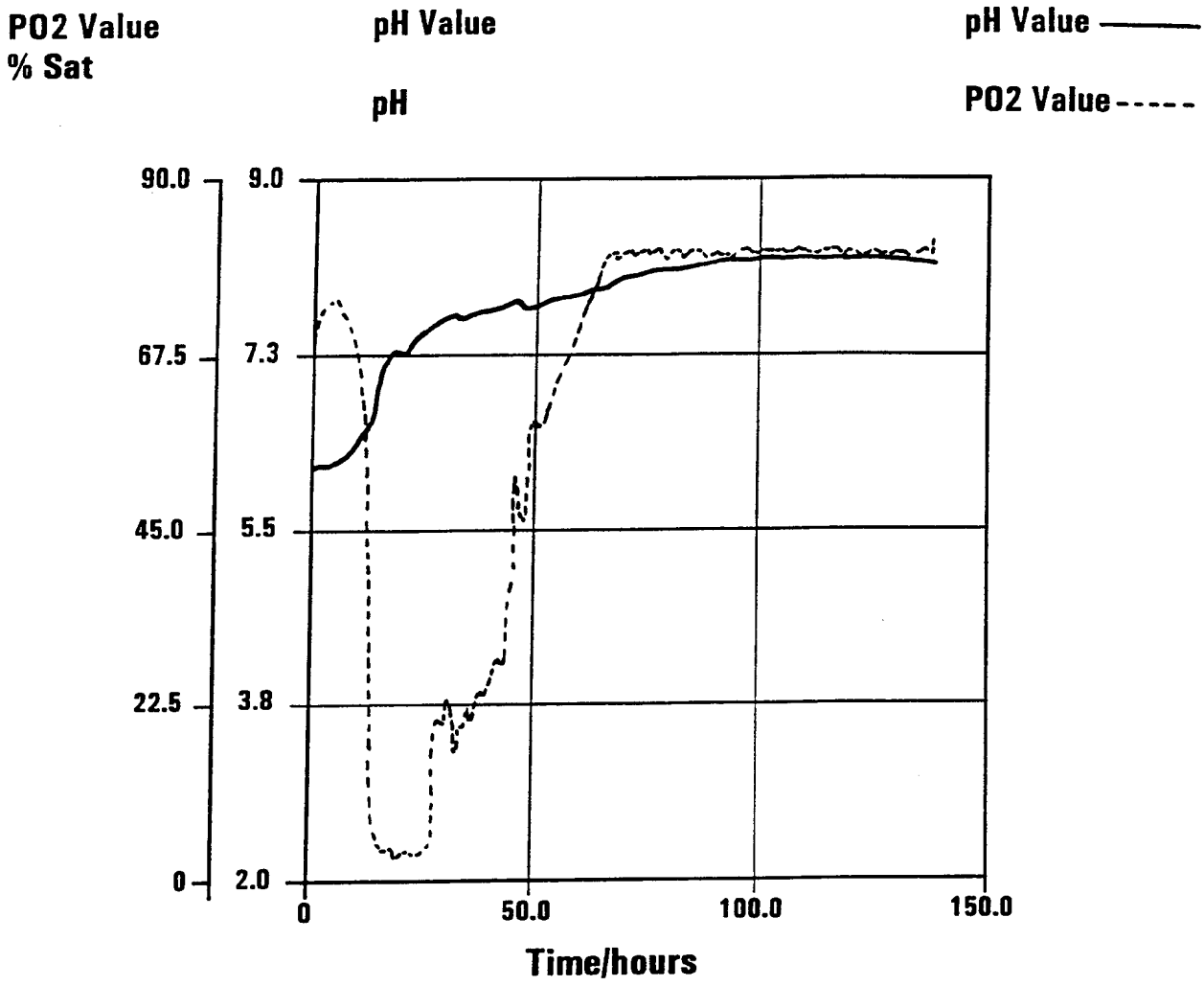

PO2 Value
% Sat
pH Value
pH
pH Value ———
PO2 Value - - - - -
90.0
67.5
45.0
22.5
0
9.0
7.3
5.5
3.8
2.0
0
50.0
100.0
150.0
Time/hours

FIG. 7

Pst I

GCT GCA GAA GTT AGA GTT GAG GGA AAC GTG CAG TTG AAT CCT ATC TTT CCA TTT GAC TTT AAG TTG

887 A E V R V E G N V Q L N P I F P F D F K L

Ssp I

GGT AGC TCT GGT GAT GAC CGT GGC AAA ATT ATT GTA ACT CAA AAC GAA AAT ATT GTT TAT

908 G S S G D D R G K I I V T Q N E N I V Y

Ase I

AAT GCT ATG TAT GAG TCT TTC TCT ATT AGC TTC TGG ATC AGA ATT AAT AAA TGG GTT TCA

928 N A M Y E S F S I S F W I R I N K W V S

AAT TTA CCA GGT TAT ACC ATC ATC GAC AGC GTT AAA AAT AAT TCA GGA TGG TCT ATT GGC

948 N L P G Y T I I D S V K N N S G W S I G

Ase I

ATA ATT AGC AAT TTT CTA GTT TTT ACA CTA AAG CAA AAC GAA AAT AGC GAG CAG GAC ATT

968 I I S N F L V F T L K Q N E N S E Q D I

SEQUENCE ID NO. 1

AAT TTT TCT TAT GAC ATT TCA AAG AAC GCT GCT GGT TAT AAT
AAA TGG TTC TTT GTA ACC
988 N F S Y D I S K N A A G Y N
K W F F V T

ATT ACC ACA AAC ATG ATG GGT AAT ATG ATG ATC TAC ATT AAC
GGA AAG CTA ATC GAC ACC
1008 I T T N M M G N M M I Y I N
G K L I D T

Age I

ATC AAA GTG AAA GAA TTG ACC GGT ATC AAC TTC TCA AAG ACC
ATT ACG TTT CAG ATG AAT
1028 I K V K E L T G I N F S K T
I T F Q M N

AAA ATC CCG AAC ACA GGA TTA ATA ACG TCT GAT TCT GAC AAC
ATC AAT ATG TGG ATT AGA
1048 K I P N T G L I T S D S D N
I N M W I R

Ase I

GAT TTC TAT ATT TTC GCA AAA GAA TTA GAT GAC AAG GAC ATT
AAT ATC CTT TTC AAT TCA
1068 D F Y I F A K E L D D K D I
N I L F N S

Sca I

CTT CAG TAC ACT AAT GTT GTT AAG GAT TAT TGG GGT AAT GAT

SEQUENCE ID NO. 1

TTA AGA TAT GAT AAG GAG

1088 L Q Y T N V V K D Y W G N D

L R Y D K E

Ase I

TAC TAC ATG ATT AAT GTA AAT TAC ATG AAT CGT TAC ATG TCT

AAA AAG GGT AAC GGT ATC

1108 Y Y M I N V N Y M N R Y M S

K K G N G I

GTC TTC AAC ACT CGT AAA AAT AAC AAC GAT TTC AAC GAA GGG

TAC AAA ATT ATC ATA AAA

1128 V F N T R K N N N D F N E G

Y K I I I K

AGA ATC CGT GGA AAT ACA AAT GAT ACT AGA GTT CGT GGT GAG

AAC GTG CTA TAT TTT AAT

1148 R I R G N T N D T R V R G E

N V L Y F N

Sca I

ACA ACG ATA GAT AAT AAG CAG TAC TCT TTA GGC ATG TAT AAA

CCG TCA AGA AAC TTA GGG

1168 T T I D N K Q Y S L G M Y K

P S R N L G

ACA GAC CTT GTT CCA TTG GGT GCA TTA GAT CAA CCG ATG GAC

GAA ATT CGT AAA TAT GGA

1188 T D L V P L G A L D Q P M D

SEQUENCE ID NO. 1

E I R K Y G

TCA TTC ATT ATT CAA CCA TGT AAT ACG TTC GAT TAC TAC GCG

AGC CAA TTA TTT CTT TCT

1208 S F I I Q P C N T F D Y Y A

S Q L F L S

Dra I

AGC AAC GCC ACG ACC AAT CGT TTA GGA ATA CTA TCA ATC GGC

TCA TAT TCT TTT AAA CTG

1228 S N A T T N R L G I L S I G

S Y S F K L

GGT GAT GAT TAC TGG TTT AAC CAT GAA TAC TTG ATC CCA GTA

ATT AAA ATC GAA CAT TAC

1248 G D D Y W F N H E Y L I P V I K I E H Y

Hind III

GCT TCA TTA CTG GAA TCA ACT TCT ACA CAT TGG GTC TTT

GTC CCA GCG TCT GAG TAA AAG CT

1268 A S L L E S T S T H W V F

V P A S E

SEQUENCE ID NO. 1

Plasmid name: abc

Version date: 10-09-1997
Version time: 12:21:18

Restriction sites found in plasmid:

Nucleotide Number	Sequence	Enzyme Name
9	ATTAAT	Ase I
56	CTGCAG	Pst I
169	AATATT	Ssp I
223	ATTAAT	Ase I
358	ATTAAT	Ase I
499	ACCGGT	Age I
558	ATTAAT	Ase I
640	ATTAAT	Ase I
719	AGTACT	Sca I
730	ATTAAT	Ase I
920	AGTACT	Sca I
1132	TTTAAA	Dra I
1258	AAGCTT	Hind III
1264	GGATCC	BamH I
1438	TCCGGA	BspE I

Expressed protein is 399 amino acids long.

Amino acid composition:

ala	10	cys	1	asp	25	glu	16
phe	24	gly	25	his	3	ile	43
lys	26	leu	26	met	12	asn	46
pro	10	gln	10	arg	14	ser	31
thr	24	val	20	trp	8	tyr	25

Total basic residues: 43
Total acidic residues: 41

SEQUENCE ID NO. 2

Plasmid: abc

```
                    Ase I
0001  CCC GCG AAA TTA ATA CGA CTC ACT ATA GGG     0030

                                        Pst I
0031  AGA CCA CAA CGG TTT CCC TCT AGT GCT GCA     0060
                                          A       0001

0061  GAA GTT AGA GTT GAG GGA AAC GTG CAG TTG     0090
0002   E   V   R   V   E   G   N   V   Q   L      0011

0091  AAT CCT ATC TTT CCA TTT GAC TTT AAG TTG     0120
0012   N   P   I   F   P   F   D   F   K   L      0021

0121  GGT AGC TCT GGT GAT GAC CGT GGC AAA ATT     0150
0022   G   S   S   G   D   D   R   G   K   I      0031

                            Ssp I
0151  ATT GTA ACT CAA AAC GAA AAT ATT GTT TAT     0180
0032   I   V   T   Q   N   E   N   I   V   Y      0041

0181  AAT GCT ATG TAT GAG TCT TTC TCT ATT AGC     0210
0042   N   A   M   Y   E   S   F   S   I   S      0051

                    Ase I
0211  TTC TGG ATC AGA ATT AAT AAA TGG GTT TCA     0240
0052   F   W   I   R   I   N   K   W   V   S      0061

0241  AAT TTA CCA GGT TAT ACC ATC ATC GAC AGC     0270
0062   N   L   P   G   Y   T   I   I   D   S      0071

0271  GTT AAA AAT AAT TCA GGA TGG TCT ATT GGC     0300
0072   V   K   N   N   S   G   W   S   I   G      0081

0301  ATA ATT AGC AAT TTT CTA GTT TTT ACA CTA     0330
0082   I   I   S   N   F   L   V   F   T   L      0091

                                        Ase
0331  AAG CAA AAC GAA AAT AGC GAG CAG GAC ATT     0360
0092   K   Q   N   E   N   S   E   Q   D   I      0101

      I
0361  AAT TTT TCT TAT GAC ATT TCA AAG AAC GCT     0390
0102   N   F   S   Y   D   I   S   K   N   A      0111

0391  GCT GGT TAT AAT AAA TGG TTC TTT GTA ACC     0420
0112   A   G   Y   N   K   W   F   F   V   T      0121
```

SEQUENCE ID NO. 2

```
0421   ATT ACC ACA AAC ATG ATG GGT AAT ATG ATG    0450
0122    I   T   T   N   M   M   G   N   M   M     0131

0451   ATC TAC ATT AAC GGA AAG CTA ATC GAC ACC    0480
0132    I   Y   I   N   G   K   L   I   D   T     0141

                                 Age I
0481   ATC AAA GTG AAA GAA TTG ACC GGT ATC AAC    0510
0142    I   K   V   K   E   L   T   G   I   N     0151

0511   TTC TCA AAG ACC ATT ACG TTT CAG ATG AAT    0540
0152    F   S   K   T   I   T   F   Q   M   N     0161

                             Ase I
0541   AAA ATC CCG AAC ACA GGA TTA ATA ACG TCT    0570
0162    K   I   P   N   T   G   L   I   T   S     0171

0571   GAT TCT GAC AAC ATC AAT ATG TGG ATT AGA    0600
0172    D   S   D   N   I   N   M   W   I   R     0181

0601   GAT TTC TAT ATT TTC GCA AAA GAA TTA GAT    0630
0182    D   F   Y   I   F   A   K   E   L   D     0191

                   Ase I
0631   GAC AAG GAC ATT AAT ATC CTT TTC AAT TCA    0660
0192    D   K   D   I   N   I   L   F   N   S     0201

0661   CTT CAG TAC ACT AAT GTT GTT AAG GAT TAT    0690
0202    L   Q   Y   T   N   V   V   K   D   Y     0211

                                         Sca
0691   TGG GGT AAT GAT TTA AGA TAT GAT AAG GAG    0720
0212    W   G   N   D   L   R   Y   D   K   E     0221

       I           Ase I
0721   TAC TAC ATG ATT AAT GTA AAT TAC ATG AAT    0750
0222    Y   Y   M   I   N   V   N   Y   M   N     0231

0751   CGT TAC ATG TCT AAA AAG GGT AAC GGT ATC    0780
0232    R   Y   M   S   K   K   G   N   G   I     0241

0781   GTC TTC AAC ACT CGT AAA AAT AAC AAC GAT    0810
0242    V   F   N   T   R   K   N   N   N   D     0251

0811   TTC AAC GAA GGG TAC AAA ATT ATC ATA AAA    0840
0252    F   N   E   G   Y   K   I   I   I   K     0261
```

SEQUENCE ID NO. 2

```
0841   AGA ATC CGT GGA AAT ACA AAT GAT ACT AGA   0870
0262    R   I   R   G   N   T   N   D   T   R    0271

0871   GTT CGT GGT GAG AAC GTG CTA TAT TTT AAT   0900
0272    V   R   G   E   N   V   L   Y   F   N    0281

                              Sca I
0901   ACA ACG ATA GAT AAT AAG CAG TAC TCT TTA   0930
0282    T   T   I   D   N   K   Q   Y   S   L    0291

0931   GGC ATG TAT AAA CCG TCA AGA AAC TTA GGG   0960
0292    G   M   Y   K   P   S   R   N   L   G    0301

0961   ACA GAC CTT GTT CCA TTG GGT GCA TTA GAT   0990
0302    T   D   L   V   P   L   G   A   L   D    0311

0991   CAA CCG ATG GAC GAA ATT CGT AAA TAT GGA   1020
0312    Q   P   M   D   E   I   R   K   Y   G    0321

1021   TCA TTC ATT ATT CAA CCA TGT AAT ACG TTC   1050
0322    S   F   I   I   Q   P   C   N   T   F    0331

1051   GAT TAC TAC GCG AGC CAA TTA TTT CTT TCT   1080
0332    D   Y   Y   A   S   Q   L   F   L   S    0341

1081   AGC AAC GCC ACG ACC AAT CGT TTA GGA ATA   1110
0342    S   N   A   T   T   N   R   L   G   I    0351

                                  Dra I
1111   CTA TCA ATC GGC TCA TAT TCT TTT AAA CTG   1140
0352    L   S   I   G   S   Y   S   F   K   L    0361

1141   GGT GAT GAT TAC TGG TTT AAC CAT GAA TAC   1170
0362    G   D   D   Y   W   F   N   H   E   Y    0371

1171   TTG ATC CCA GTA ATT AAA ATC GAA CAT TAC   1200
0372    L   I   P   V   I   K   I   E   H   Y    0381

1201   GCT TCA TTA CTG GAA TCA ACT TCT ACA CAT   1230
0382    A   S   L   L   E   S   T   S   T   H    0391

                                          Hind
1231   TGG GTC TTT GTC CCA GCG TCT GAG TAA AAG   1260
0392    W   V   F   V   P   A   S   E
```

SEQUENCE ID NO. 2

IIIBamH I

1261 CTT GGA TCC GAG CTT GAG TAT TCT ATA GTG 1290

1291 TCA CCT AAA TCC CAG CTT GAT CCG GCT GCT 1320

1321 AAC AAA GCC CGA AAG GAA GCT GAG TTG GCT 1350

1351 GCT GCC ACC GCT GAG CAA TAA CTA GCA TAA 1380

1381 CCC CTT GGG GCC TCT AAA CGG GTC TTG AGG 1410

BspE

1411 GGT TTT TTG CTG AAA GGA GGA ACT ATA TCC 1440

SEQUENCE ID NO. 2

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IB 99/01301

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/33 C12N15/31 C12N15/75 C12N1/21 A61K39/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 08540 A (OPHIDIAN PHARMACEUTICALS) 5 March 1998 (1998-03-05)	1,2,5-32
Y	page 7, line 6 - line 19 page 20, line 26 - page 21, line 29 page 23, line 14 - line 15 page 24, line 5 - line 18 page 35, line 3 - line 11 page 37, line 25 - line 29 page 38, line 19 - line 20 claims 1-18; example 47	3,4

Y	MORIISHI K ET AL: "Mosaic structures of neurotoxins produced from Clostridium botulinum types C and D organisms" BIOCHIMICA BIOPHYSICAL ACTA, vol. 1307, 1996, pages 123-6, XP000857076 the whole document	3,4

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1999	15/12/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Le Flao, K

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/IB 99/01301

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 96 41881 A (MICROBIOLOGICAL RESEARCH AUTHORITY) 27 December 1996 (1996-12-27) claims 1-23	1-32
A	WO 98 07864 A (MICROBIOLOGICAL RESEARCH AUTHORITY CAMR) 26 February 1998 (1998-02-26) claims 1-52	1-32
A	US 4 292 307 A (VALENTINA P ZEMLYAKOVA) 29 September 1981 (1981-09-29) column 2, line 50 - line 62 column 4, line 50 -column 5, line 53; claim 7	1-32

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INTERNATIONAL SEARCH REPORT

Ir. ..ational application No.
PCT/IB 99/01301

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claim 19 is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 20-32
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

Inter· nal Application No
PCT/IB 99/01301

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9808540 A	05-03-1998	AU 4245097 A	19-03-1998
WO 9641881 A	27-12-1996	AU 705944 B	03-06-1999
		AU 6012196 A	09-01-1997
		CA 2224444 A	27-12-1996
		EP 0833919 A	08-04-1998
		JP 11507827 T	13-07-1999
WO 9807864 A	26-02-1998	AU 4389597 A	06-03-1998
		EP 0939818 A	08-09-1999
US 4292307 A	29-09-1981	NONE	